Annals of Clinical and Analytical Medicine
Original Research

Key issues in tracheal injuries and complications

Tracheal injuries

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This case was presented as a poster presentation at the 22nd Annual Meeting of the Asian Society for Cardiovascular and Thoracic Surgery ASCVTS 2014 3–6 April 2014 Istanbul, TURKEY

Abstract
Aim: In this study, we aimed to investigate the histopathological changes in tracheal epithelia after conventional tracheostomy in a rabbit model.
Material and Methods: Thirty-nine male New Zealand White rabbits were used for this study. They were randomly divided into five groups. Classic tracheostomy was carried out in 29 subjects. Group 1, which was created after any surgical procedure, served as a control group. After tracheostomy, we applied sterile saline in Group 2, Mitomycin-C (MMC) (0.8 mg/ml) in Group 3, MMC (0.4 mg/ml) in Group 4 and 5-Fluorouracil (5-FU) (10 mg/ml) in Group 5 around tracheotomy for 5 minutes.
Results: At three weeks after surgery, tracheas were evaluated by morphometric and histopathological examination, including tracheal lumen diameter, the number of capillary vessels, subepithelial tissue thickness, fibroblasts, and inflammatory cells. There were statistically significant differences between tracheostomy and control group for tracheal lumen diameter (P=0.35), number of capillary vessels (P=0.06), subepithelial tissue thickness, fibroblasts and number of lymphocyte ($p < 0.001$). Histopathological analysis showed decreased fibrosis in both of the groups treated with MMC and 5-FU.
Discussion We believed that wound healing modulation may prevent scar formation, but requires further treatment. Herein, as we have shown in our study, fibroblasts are critical cells, but not one. MMC and 5-FU are medications that have been used for antiproliferative activity on fibroblasts.

Keywords
Tracheal epithelia; Mitomycin-C; 5-Fluorouracil

DOI: 10.4328/ACAM.20492 Received: 2021-01-18 Accepted: 2021-02-21 Published Online: 2021-02-23 Printed: 2021-04-01 Ann Clin Anal Med 2021;12(4):433-437
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Introduction

However, tracheostomy is associated with a considerable morbidity, with reported complication rates of 8–45% [1,2]. Its complications range from intraoperative that can be rapidly life-threatening, to late or chronic postoperative, which may become apparent much later, such as tracheal stenosis (TS) and granulation [3]. Benign tracheal stenosis usually depends on prolonged endotracheal intubation (ETI) and tracheostomy. Clinically significant stenosis after prolonged intubation is very rare and occurs in approximately 1% of patients. They arise due to local ischemic necrosis of the tracheal wall due to defects in the new scar formation [4].

Mitomycin-C (MMC) is an antitumor antibiotic derived from Streptomyces caespitosus. It has an antineoplastic effect that causes breakage in the cross-linking of DNA like alkylating agents, at higher concentrations; it inhibits the protein and RNA synthesis. Similarly, 5-Fluorouracil (5-FU) is a pyrimidine analogue with antimetabolic activity due to blocking the enzyme thymidylate synthase, which converts ribonucleotides deoxyribonucleotides, thus inhibiting DNA synthesis. MMC and 5-FU are medications that have been used for antiproliferative activity on the fibroblasts [5].

We aimed to investigate the histopathological changes in tracheal epithelia caused by the application of topical MMC and 5-FU after the conventional tracheostomy in a rabbit model.

Material and Methods

Thirty-nine male New Zealand White rabbits, 24 weeks of age were used for this study. They were randomly divided into five groups. All animals received humane care and were used in compliance with the standards established by the European Convention for Animal Care and Use of Laboratory Animals. The rabbits were fed a standard pelleted diet, and tap water was provided ad libitum as drinking water. Animals were housed in conventional individual cages on a 12-hour light/dark cycle at room temperature in a humidity-controlled environment. This experiment was approved by the local animal care and the local ethics committee for animal experiments.

Groups

The subjects were divided into five groups: control containing ten and tracheostomy eight rabbits. Group 1, which was created after any surgical procedure, served as a control for tracheal measurements. After tracheostomy, we applied sterile saline in Group 2, MMC (0.8 mg/ml) (Mitomycin- C Kyowa 10 mg /flk, Kyowa Hakko Kogyo Ltd. Tokyo, Japan) in Group 3, MMC (0.4 mg/ml) in Group 4 and 5-FU (10 mg/ml) (5-fluorouracil 1000 mg/20 ml flk, Sandoz Pharmaceutical Company, Istanbul, Turkey) in Group 5 around tracheotomy for 5 minutes.

Anesthesia

Ketamine HCl induced general anesthesia (Ketanest, Pfizer Pharma GmbH, Karlsruhe, Germany) 15-20 mg/kg i.v. or 20-25 mg/kg i.m. in addition was maintained by Xylazine (Alfazyne 2%; Alfasan International. BV, Woerden, Holland) 0.5-1 mg/kg i.v. or 1-2 mg/kg i.m. Previous doses were repeated with a reflex response to Ketamine HCl and Xylazine for the depth of anesthesia as needed.

Operation technique

Under general anesthesia, all subjects were placed in a supine position on the operating table with spontaneously breathing. Traditional tracheostomy was performed in 32 subjects. Group 1, which any surgical procedure was made, served as a control for tracheal measurements. After tracheostomy, all subjects immediately received the research medication applications with cottoned pledget topically to the tracheotomy site around the tube. We applied sterile saline in Group 2, MMC (0.8 mg/ml) in Group 3, MMC (0.4 mg/ml) in Group 4 and 5-FU (10 mg/ml) in Group 5 around tracheotomy for 5 minutes.

Postoperative Care and Follow-Up

Tradomol HCl (Contramal, 100 mg 2 ml, Abdi Ibrahim Ltd., Istanbul, Turkey) 2 mg/kg/day i.m. from the first to the fifth day was used for pain control. The animals were followed-up for about three weeks after surgery. All animals were euthanized with a lethal IV dose of non-barbiturate anesthetic (Ketamine/Xylazine) painlessly according to the existing instructions established by the latest AVMA Panel report on Euthanasia [6].

Pathological Evaluation

All materials were fixed in 10% of buffered paraformaldehyde for 48 hours. Tissue specimens were prepared in an automatic tissue processor (Leica ASP300 S-Tissue Processing), embedded in paraffin and sectioned with a microtome (Leica RM 2025 microtome). The sections (5μm) were stained in an automated slide processing system (VENTANA BenchMark XT Automated Slide Processing System) with Hematoxylin and Eosin and Masson's trichrome. A Nikon Eclipse E400 light microscope (Nikon Corporation. Minatoku, Tokyo Japan) investigated the stained specimens at the 20-fold magnified area in tissue samples. For each sample, the same area was photographed after staining with a Nikon Coolpix 5000 camera (Nikon Corporation. Minatoku, Tokyo Japan) attached to a microscope. In the pathological evaluation, depending on the magnification used, the photograph of Nikon micrometer microscope slide (MBM11100, Nikon Corporation. Minatoku, Tokyo Japan) was also taken for calibration. All images were then transferred into a PC environment and analyzed using the Clemex Vision Lite 3.5 Image Analysis program. The length was calibrated by comparing the specimen's photograph with a Nikon micrometre's photo microscope slide, which was taken under the same magnification. Areas of 0,25 square millimeters were designated using Clemex Vision Lite 3.5 (Clemex Technologies inc. Longueuil, Quebec Canada) Image Analysis program. Fibroblasts, capillary vessel, and lymphocytes were labelled and counted with the same Image Analysis program in an area of 0,25 square millimeters. The damaged cells were excluded from the study. Marked cells were counted with the same computerized Image Analysis Program without human intervention. Tracheal lumen diameter (Figure 1A) and subepithelial tissue thickness (Figure 1B) were also measured with the same Image Analysis program. The pathologist evaluated the sample blindly to its origin.

Statistical analysis

Results were analyzed using the SPSS 18.0 portable for Windows (SPSS Inc, Chicago, Illinois, USA). Data noted in the third week were expressed as median and interquartile range (25%-75% quartiles). The Kruskal-Wallis test analyzed the comparison between groups. The Mann– Whitney U-test was used to compare the significance between the control and

MMC / 5-FU groups. A p-value <0.05 was used to indicate a significant difference.

Results

Only three animals were excluded from the study due to necrosis. Regardless of the cause of death, dead animals are replaced with new ones. Thirty-nine subjects were included in our experiment also lived up to the end of three weeks. There was no skin reaction, wound infection, or bleeding around the surgical site in the perioperative period. No local complications were encountered connected with MMC or 5-FU.

A statistically significant difference ($p<0.035$) was found between groups of Tracheal Lumen Diameter (TLD). In the control group, Group 1, TLD was 3198.8 (3027.3-3926.9), the maximum TLD loss of 2530.3 (1415.3-2897.5) was detected in Group 2 with a sterile saline group, 2616.4 (2338.1-2832.5) in Group 4 underwent 0.4 mg/ml MMC, the closest values to Group 2 were obtained. In 0.8 mg/ml MMC Group 3 and 10 mg/ml 5-FU group 5, 3243.3 (2148.6-3771.9) and 3190.9 (2216.7-3767.7) were found, respectively (Figure 2A). High-doses of MMC and 5-FU were close to each other, but the best result was in Group 5.

There was a statistically significant difference ($p<0.006$) in subepithelial tissue thickness (STT) between the groups. In the control group, STT was 168.3 (94.3-281.1). The highest increase in STT was in Group 2: 516.1 (327.3-741.1) followed by Group 4: 433.8 (381.3-493.4), Group 5: 428.7 (243.2-635) and Group 3: 298.9 (243.9366), respectively. According to the control group, Group2, 3, 4, and five increased threefold by1.7 times, 2.57 times, and 2.54 times, respectively (Figure 2B).

A statistically significant difference ($p<0.001$) was found in inflammatory cells (IC) between groups. Ten (6.5-16) ICs were detected in the control group. The highest increase in IC was in Group 2, 229.5 (48.5-454), followed by Group 4, 54.5 (35.5-73.5), Group 3, 34 (30.5-39) and Group 5, 22 (18-32) cells (Figure 3A). According to the control group, in groups 2, 3, 4, and 5, it increased 23-fold, 3.4-fold, 5.4-fold, and 2.2-fold, respectively.

A statistically significant difference ($p<0.001$) was found in Capillary Vessels (CV) between the groups. In the control group, 4 (2-6) CVs were detected. The highest increase in CV was in Group 2, 19 (15-29.5), followed by Group 4, 15.5 (9-37.5), Group 3, 8 (6-9) and Group 5, 6 (4-10) capillary vessels (Figure 3B). According to the control group, groups 2, 3, 4, and 5, it increased five times, twice, 3.9 times and 1.5 times, respectively.

A statistically significant difference ($p<0.001$) was found in fibroblasts between the groups. In the control group, 5 (3,5-8) fibroblasts were detected. The highest increase in fibroblasts was in Group 2, 30.5 (21-36), followed by Group 4, 19 (13.5-28.2), Group 5, 12 (12-16) and Group 3, 10 (8.5-14) fibroblasts (Figure 3C). According to the control group, groups 2, 3, 4, and 5, it increased six-fold, two-fold, 3.8- fold, and 2.4- fold, respectively.

5-FU is more effective than MMC in TLD, IC, and CV. MMC was only 0.84 fold more evident on STT and 0.4 times less effective on Fibroblasts.

Discussion

TS is a significant decrease in the tracheal lumen that can be potentially life-threatening and avoided at large. A cascade of pathological events started with pressure and/or excessive irritation ulceration and subsequent healing, starting with granulation, progressing to cicatrization and scar contraction [6]. They ordinarily occur at the tracheostomy or ETI tube cuff site and less commonly at the end of the cannula.

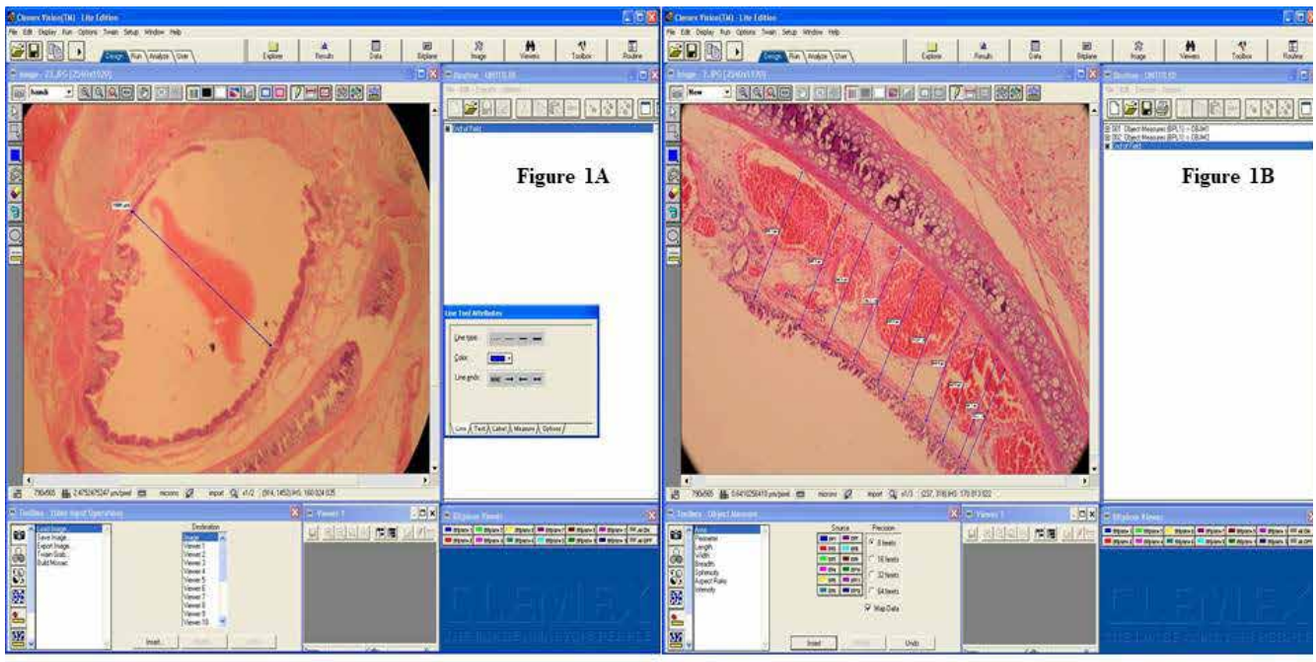

Figure 1A. Tracheal lumen diameter is measured at the 20-fold magnified area in tissue samples by Clemex Vision Lite 3.5 Image Analysis system ®. The measurement is made on the narrowest line in the midline.

1B. Subepithelial tissue thickness is measured at the 20-fold magnified area in tissue samples by the same image analysis system. The thickness is measured in a few places during the measurement, and the average is calculated automatically by Clemex Vision Lite 3.5 Image Analysis system ®.

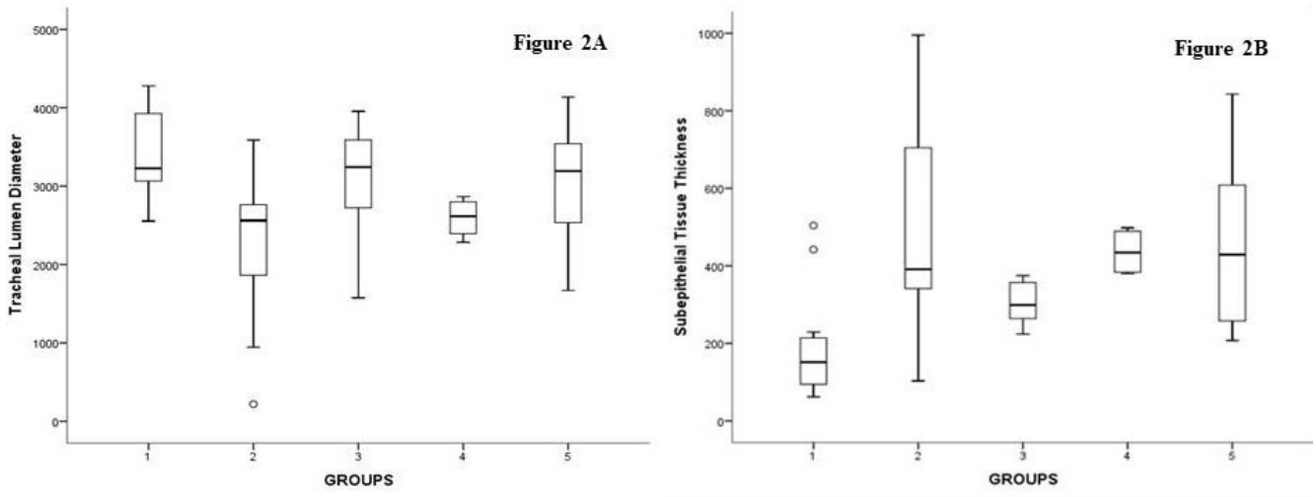

Figure 2A. Tracheal Lumen Diameter comparison between experimental groups
2B. Subepithelial Tissue Thickness comparison between experimental groups

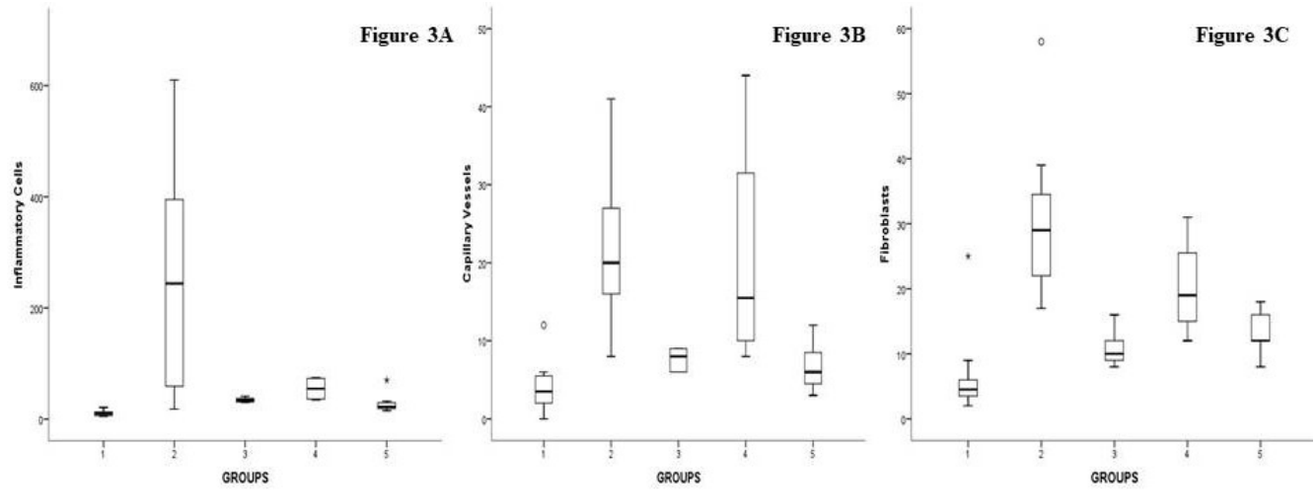

Figure 3A. Inflammatory Cells comparison between experimental groups
3B. Capillary Vessels comparison between experimental groups
3C. Fibroblast comparison between experimental groups

Although ETI has serious complications, it has been ignored because of its life-saving feature. In the literature, these complications are considered in studies with less than one hand fingers. Unfortunately, tissue damage and granulation can occur even within hours. Esteller et al. showed laryngotracheal injury in 11%, and Vallés et al. in 8.1% of the patients who underwent ETI [7]. In the same study by Esteller et al., severe TS was found as 3.1%, and when lower TSs were added, it was perceived as 4.6%. In another study, it was reported that TS was about 6-21%. With increasing intubation, tracheostomy, indications and possibilities of mechanical ventilation, prolonged survival and the increased number of days spent in ICUs led to an increase in TS incidence. In the United Kingdom, 30.000 patients undergo mechanical ventilation, and 500.000 in the United States annually. This means 1.380 new patients in the UK and 23.000 in the USA [8]. The incidence may even differ between countries and even between regions in the same country.

Thankfully, clinically significant TS develops in about 1% of patients after tracheostomy or ETI [9]. Several treatments have been used for its treatment; current modalities tend to fail due to the new scar formation and restenosis through either the persistence of the chronic inflammatory process that caused the initial stenosis or the surgical intervention [5]. In the literature, because of the effect of topical MMC and 5-FU on fibroblast proliferation in TS, many in vitro and in vivo studies are available. However, Wang et al. have not recommended for use, since they increase anastomosis complications after 263-disease current severe resection [10]. In another study, Gangar et al. have been using it for 16 years for the same purpose, but it has not proven useful. As a result, the utility remains hypothetical, and its future role is unclear [11].

Wang et al. evaluated 263 patients with idiopathic subglottic stenosis (ISS) and found that 47% of antinuclear antibodies were positive. Tracheal and endoscopic procedures have been applied to 92% of cases before. In 97% of the cases, tracheal membranous wall flap resection and tailored cricoplasty were performed. In 23 patients (8.7%), 14 of whom were mild and nine were persistent, recurrences requiring dilatation were detected. Steroids, mitomycin C use, and previous tracheostomy, stents,

and vocal cord involvement have been found as anastomotic complications and risk factors for recurrence. In the study published in 1993, the first phase of which lasted for 42 years, the average diagnosis was 36 months, and the duration until surgical treatment was 24 months; 42% of the patients were treated with asthma misdiagnosis. After 1998, there has been an increase together with decreases in some periods. MMC was administered to 27 patients (11.4%) in the form of endobronchial injection without specifying the dose and duration of administration, and the steroid was administrated to 12 patients (5%). The results that we extracted from this study are that surgery is the best treatment method for the selected patients at the right time and that MMC has been administered to only 27 patients in 40 years, and that they can lead to anastomotic complications with steroids parallel to the literature [10].

Gangar et al. studied pediatric airway diseases, and in a randomized, double-blind, placebo-controlled study, they applied 0.4 mg/ml MMC for 2 minutes to 24 patients with grade 3 or 4 stenosis and underwent laryngotracheal reconstruction or cricotracheal resection. It was terminated upon its invalidity [12]. In a similar study, 0.5 mg/ml topical MMC was administered to 26 patients single and twice; the twice application was found to be more beneficial. However, this benefit was only 3.8 years for dual applications and 2.4 years for unique applications, not five years. Also, Hseu et al. reported that 66 of 92 adults showed no benefit in using MMC, and indeed, MMC receivers showed a second procedural requirement. However, the authors attribute this to their use in more severe and difficult situations [12, 13]. As previously demonstrated in our current study [5], when MMC was used at low doses such as 2 minutes and 0.4-0.5 mg/ml, no benefit could be detected. In Group 4, where 0.4 mg/ml MMC was used, only fibroblast and IC were reduced compared to Group 1, which underwent tracheostomy; despite a relative decrease in TLD, STT and CV, similar values were obtained. There is a statistically significant difference with the control group.

There is no consensus on the treatment dose of MMC. The majority of human studies use a topical dose of 2-10 mg/ml due to ophthalmological experience, and 0.1-0.4 mg/ml in animal studies. However, we agree that humans can safely use and heal in a wider field on less sensitive tissues, repeated doses at higher concentrations [12]. In vitro proliferation of MMC in human fibroblasts at 1.6 mg/ml and increased level showed inhibition. This effect of MMC increases with dose, and in an animal model, in vivo cultures and airway, it is limited to 3 weeks and four weeks in the skin [14].

To prevent the formation of scar tissue, MMC and 5-FU were used in our experiment. According to the literature, there were statistically significant differences between tracheostomy and control group for TLD (P=0.35), CV (P=0.06), STT, fibroblasts, and lymphocyte count ($p < 0.001$).

In conclusıon, they are currently successfully used as adjunctive therapeutic agents to prevent excessive scarring in various surgical fields, including ophthalmology, otorhinolaryngology, and urology. The works showing the usefulness are presented in retrospective and case presentations. A low-dose and a small number of patients have been used in very few prospective studies. Therefore, their effects have not been shown. We think it is too optimistic to believe that it will be prevented with a single medication dose. We still believe that wound healing modulation may prevent scar formation and need further treatment. Herein, as we have shown in our study, fibroblasts are critical cells, but not one. MMC and 5-FU are medications that have been used for antiproliferative activity on the fibroblasts.

Acknowledgment
We are grateful to the pathologist, Prof. Mustafa Cihat Avunduk M.D. from the Department of Pathology, for her contribution and assistance in evaluating statistical and pathological analysis.

Scientific Responsibility Statement
The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement
All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

Funding: *None*

Conflict of interest
None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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How to cite this article:
Mustafa Calik, Saniye Goknil Calik. Key issues in tracheal injuries and complications. Ann Clin Anal Med 2021;12(4):433-437